Preclinical and clinical studies on the co-regulation of tumor-induced angiogenesis and dendritic cell suppression

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CHAPTER 9

Summarizing discussion and future perspectives

Over the past two decades, new anti-cancer therapeutics have been developed based on an increased understanding of tumor biology. For tumor types with a limited number of unique alterations, a treatment specifically targeting those alterations should be developed, e.g. imatinib targeting bcr-abl or cKIT in chronic myeloid leukemia and gastro-intestinal stroma cell tumors, respectively. In patients, who have tumor types with alterations in multiple pathways, such as breast cancer and the vast majority of NSCLC, we should try to design anti-cancer strategies that target multiple pathways driving the biology of these tumors in order to reduce acquired resistance and improve outcome. In the introductory **chapter 1** of this thesis, we describe two general features of virtually all solid tumors that contribute to tumor progression, i.e. angiogenesis and immune escape. The cross-talk and inverse co-regulation between angiogenesis and the immune response are reminiscent of the physiological mechanisms underlying wound healing. The continuous production of growth signals by tumors leading to ongoing angiogenesis have contributed to the notion that tumors are wounds that never heal (1). Ongoing angiogenesis interferes with infiltration of immune cells into the tumor via decreased expression of adhesion molecules on endothelial cells, a process called tumor endothelial cell anergy (2). The contribution of infiltrating immune cells to tumor progression has been a matter of discussion (3). Tumors have the ability to alter normal hematopoiesis and DC differentiation via tumor-derived factors, leading to accumulation of immature myeloid-monocytic cells, which may aid tumor development by release of pro-angiogenic factors or transdifferentiation to endothelial-like cells, both in support of neovascularization (4). These so-called myeloid suppressor cells (MSC) also exert a profound inhibitory activity on the anti-tumor immune response (5). Therefore, MSCs may be a target for modulation of both angiogenesis and the immune response. All-*trans* retinoic acid (ATRA) and 1,25-hydroxyvitamin D3 have been shown to reduce MSC frequencies in preclinical models directly and indirectly, respectively (6-8). In addition, STAT3 inhibition may allow differentiation of hematopoietic cells into fully mature DCs in the presence of tumor-derived suppressive factors, and thus prevent induction of MSC (9). We also observed a reduction in MSC frequencies in advanced cancer patients treated with cediranib, a specific VEGFR inhibitor (chapter 5). This is consistent with the notion that some tumor-derived factors, such as VEGF and gangliosides, interfere with a proper hematopoiesis and DC differentiation, while simultaneously stimulating angiogenesis (figure 1). This inverse co-regulation between tumor-induced angiogenesis and immune suppression was the main subject of study in this thesis with the express outlook that an increased understanding of the factors and mechanisms underlying this co-regulation might translate into more effective anti-cancer strategies.

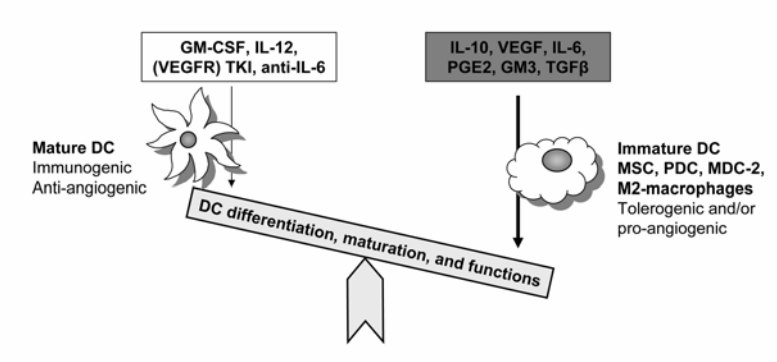

Figure 1 - *The balance between DC maturation and suppression and possible consequences for immunity and angiogenesis. Figure represents situation in cancer-conditioned environement.*

In **part I** of this thesis we describe the current clinical experience with VEGFR TKIs. VEGF is widely regarded as an important tumor-derived modulator of both angiogenesis and DC differentiation. Interference with VEGFR signaling might therefore affect both processes. Although anti-angiogenic treatment via TKIs is widely applied in the clinic, there are still issues and questions with respect to toxicity and efficacy that remain to be elucidated. Since angiogenesis is supposed to be quiescent during adult life and only activated in tumoral areas, it has been suggested that targeting angiogenesis as a therapeutic anti-cancer strategy would cause little toxicity. Nevertheless, clinical administration of VEGFR TKI has uncovered very particular side effects. **Chapter 2** outlines the specific toxicities of VEGFR TKIs and their management as well as issues regarding acquired resistance and tumor evaluation. Moreover, the need for a better understanding of long-term effects of VEGFR TKIs on tumor biology and normal human cell and tissue physiology is discussed. In phase I clinical studies, in which pharmacodynamics of new agents are explored, patients are monitored for a relatively short period. Since the consequences of anti-angiogenic treatment such as with VEGFR TKI change over time (10;11) and the treatment will be administered over long periods (preferably chronically), patients should be monitored beyond the first months of treatment. Pharmacodynamics should therefore also be included in phase II or even phase III studies. As repetitive tumor biopsies are not feasible in the clinic, identification of systemic biomarkers, such as serum VEGF levels or soluble VEGFR-2 levels, is important. These can help to monitor, non-invasively and on a large-scale, long-term effects of anti-angiogenic agents. Understanding the long-term effects of these TKIs may lead to optimized administration schedules with a reduced chance of inducing acquired resistance and may help to design better combination regimens that finally will improve efficacy. One such possible regimen is the combination of anti-angiogenic TKI therapy with inhibition of the EGFR pathway. **Chapter 3** describes

the rationale to combine these two strategies: preclinical evidence shows an important role of the EGFR pathway in angiogenesis, either directly by stimulating endothelial cells or indirectly by tumoral production of angiogenic molecules upon EGF stimulation. Additionally, acquired resistance to EGFR inhibitors can be circumvented by angiogenesis inhibitors. Results of a phase I study combining cediranib, a VEGFR TKI, and gefitinib, an EGFR TKI, are reported in **chapter 4**. This study showed that combination treatment was generally well tolerated with manageable adverse events. The most common adverse events were diarrhea, hypertension, anorexia and fatigue. The protocol-defined maximum-tolerated dose (MTD) of cediranib was 30 mg/day with gefitinib 250 mg/day. In a parallel expansion cohort, 45 mg/day cediranib was the maximum investigated dose combined with gefitinib 500 mg/day and found to be tolerated well. Since 45mg/day was previously found to be the MTD for cediranib monotherapy, no further expansions beyond this dose were investigated. Pharmacokinetics of both cediranib and gefitinib were not substantially affected when administered in combination. Encouraging evidence of anti-tumor activity was observed as 9% of the patients experienced a partial response. Of special interest were patients with advanced RCC; 33% of advanced RCC patients achieved a partial response. The results of this phase I study support the rationale as discussed in chapter 3 for concurrent inhibition of the VEGFR and EGFR signaling pathways.

As mentioned above and discussed in chapter 1, there is increasing evidence of co-regulation of angiogenesis and defective DC differentiation in cancer patients. In **part II** we explored whether targeting angiogenesis via VEGFR TKI could affect tumor-induced DC suppression, providing a window for combined immunotherapy. In **chapter 5** we investigated whether clinical VEGFR inhibition by cediranib could overcome tumor-induced suppression of DC differentiation. Cediranib also inhibits to a lesser extent TK activity of PDGFRs and cKIT. We observed decreased frequencies of myeloid and plasmacytoid DCs and increased frequencies of MSCs in the peripheral blood of advanced cancer patients as compared to healthy donors. The effect of advanced tumors on DC differentiation is therefore systemic and results in a profound reduction of more mature DC in the circulation and a simultaneous accumulation of immature myeloid DCs with a potentially immunosuppressive role. Although tumor-derived VEGF is thought to be a major factor involved in DC suppression (12;13), one-month treatment with cediranib could not restore the aberrant DC (precursor) frequencies. It did, however, result in a trend towards reduced MSC frequencies. In advanced cancer patients DC suppression is most likely caused by multiple and/or tumor-specific factors and cediranib may have been too selective for VEGFR inhibition in this regard. In **chapter 6** we investigated whether sunitinib, targeting not only the tyrosine kinase activity of all three VEGFRs, but also the tyrosine kinase activity of the PDGFRs, fms-like tyrosine kinase-3 (FLT3), and cKIT, could normalize the aberrant myeloid lineage differentiation profile in advanced RCC patients. Our data showed that daily administration of sunitinib 50 mg for 4 weeks resulted in a

combined lowering of abnormally high levels of immunosuppressive myeloid leukocytes and a relative increase of immunostimulatory CD1c$^+$ MDC-1 frequencies, the latter specifically in patients with tumor regression. Apart from the use of different agents with their own inhibitory profile with the more promiscuous sunitinib targeting a wider range of cytokine and growth factor receptors, the discrepancy between the results described in chapters 5 and 6 could also be explained by the inclusion of multiple cancer types, with their own specific set of tumor-derived factors, in the study described in chapter 5 as opposed to just RCC in chapter 6.

In these studies we investigated the frequencies and phenotype of three major DC subsets recognized in human peripheral blood: the conventional myeloid MDC-1 (CD1c/BDCA-1$^+$) and MDC-2 (CD141/BDCA-3$^+$) subsets and the CD303/BDCA-2$^+$ plasmacytoid DC (PDC) subset. While the relative contribution and importance of these subsets to the generation of an anti-tumor immune response is still largely unknown, important clues are starting to surface. While PDC can contribute directly to the priming of anti-tumor effector cytotoxic T lymphocytes, either through type-I IFN production or through antigen presentation, MDC are generally found to be more powerful in this regard. In addition, tumor-conditioned PDC have been shown to have pro-angiogenic features (14). Differential expression of TLR and C-type lectins on MDC-1 and MDC-2 suggests distinct functions of these two subsets (15-17). Intriguingly, transcriptional profiling data indicated BDCA-3/CD141$^+$ DC to be akin to the murine CD8α$^+$ DC subset and suggested that they might be involved in cross-tolerance or cross-priming, similarly to this specialized murine subset (17). Recently, a role for the MDC-2 subset has been suggested in Th2-skewing as the MDC-2 population is increased in atopic patients and CD141/BDCA-3$^+$ MoDC preferentially induce T cells with a type-2 cytokine profile (18). In contrast, MDC-1 have been shown to secrete high levels of the type-1 T cell-skewing IL-12 upon appropriate stimulation (19). The latter would thus clearly favor the generation of effective anti-tumor immunity. Accordingly, we showed that MDC-1 frequencies, but not MDC-2 frequencies, were positively associated with prolonged PFS in sunitinib-treated RCC patients (chapter 6). Indeed, in stark contrast to MDC-1, we observed a trend for high (i.e. above median) MDC-2 frequencies to be associated with a reduced PFS in the same patients (see chapter 6, figure 5d). This would be in keeping with a tolerizing or Th2-skewing capacity of MDC-2. In this regard, it is of particular interest that an IL-10 induced subset of monocyte-derived DC with an immune-suppressive phenotype was previously shown to express the MDC-2 associated marker thrombomodulin (i.e. CD141/BDCA-3) (21). CD141 as a marker of tumor-conditioned immunosuppressive DC is currently under further investigation in our lab.

Based on the results reported in chapter 6, we hypothesize that decreasing immune suppressive factors and/or the responsiveness of DC precursors (in particular MDC-1) to these factors, might improve the efficacy of immunotherapeutic approaches. To our knowledge only one clinical study employing the combination of anti-angiogenesis and

vaccination has been reported so far: in hormone-refractory prostate cancer patients treated with DC-based vaccines and bevacizumab (i.e. anti-VEGF monoclonal antibody), PSA responses and immune responses were observed (22). Based on our findings sunitinib is also a promising candidate in this regard. Indeed, the beneficial pro-immune effects of this TKI are not limited to DC differentiation, but have also been demonstrated in regard to regulation of Tregs, the frequencies of which were shown to decline in RCC patients upon sunitinib treatment, concurrent with increased type-1 T cell reactivity (23). In a very small pilot experiment, we found decreased Treg frequencies in only one out of four patients receiving sunitinib (figure 2). Interestingly, the one patient in who this reduction in Treg rates was observed underwent a partial response (PR). We thus obtained anecdotal evidence that Treg rates might decline upon sunitinib administration in relation to an apparent clinical benefit from the treatment. Certainly this promising link between sunitinib treatment and a decrease in Treg numbers warrants further investigation, both phenotypically and functionally, in expanded patient groups.

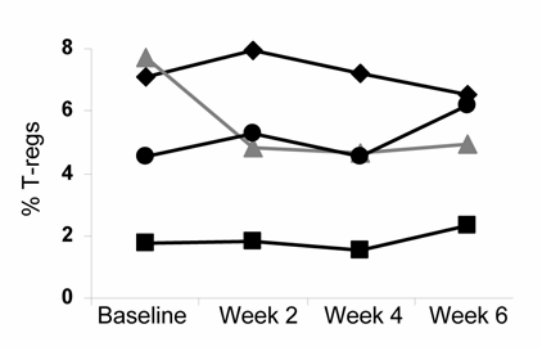

Figure 2 *- $CD25^{hi}FoxP3^{+}$ T-regulatory cells (Tregs) as percentage of $CD3^{+}CD4^{+}$ T cells in four renal cell cancer patients over the course of treatment with sunitinib for four weeks, followed by a 2-week off period (between weeks 4 and 6). Gray line represents patient experiencing a partial tumor response.*

In **part III** we further examined tumor-induced DC suppression in preclinical studies to obtain clues for viable targets to develop more effective anti-cancer strategies. In **chapter 7** we studied the newly recognized therapeutic targets ganglioside GM3 and STAT3 as modulators of both angiogenesis and immunity. We investigated immunohistochemically in a tissue micro arraywhether these markers were associated with inhibition of DC activation (number of $CD1a^{+}$ vs $CD83^{+}$ DC) and angiogenesis (microvessel density by $CD34^{+}$ staining) in NSCLC (n=176). Our studies showed that both GM3 and phosphorylated STAT3 (pSTAT3) were widely expressed in NSCLC and that GM3 expression was associated with a favorable patient survival, although this did not reach statistical significance. pSTAT3 expression could not be associated with either suppressed DC activation or

angiogenesis. In addition, we did not find evidence for a pro-angiogenic role of GM3. We could, however, confirm a possible involvement of GM3 in tumor-induced DC suppression in NSCLC patients as decreased numbers of tumor-infiltrating mature DC were observed in tumors with high GM3 expression. Its high tumor-specific expression makes GM3 a possible candidate for tumor targeting. Approaches to target gangliosides are currently in early clinical development. Many studies focus on monoclonal antibodies including antibodies against *N*-glycolyl-containing gangliosides (i.e., 1E10) and on vaccine-based strategies (24-27). The more specific murine monoclonal antibody 14F7, which we used in our study, recognizes *N*-glycolyl GM3 and has entered clinical development (28). Although induced immune responses have been reported, data on clinical efficacy are still lacking. Our findings in chapter 7 suggest that, beside angiogenic effects, it might also be worthwhile to monitor immune effects of therapeutic strategies targeting GM3.

Chapter 8 concerns an additional immunotherapeutic target, IL-6, in the biology of glioblastoma. In an *in vitro* DC differentiation model, using the human acute myeloid leukemia cell line MUTZ-3, glioblastoma-conditioned medium prevented $CD34^+$ precursors to acquire DC and LC characteristics and functions, and concomitantly induced STAT3 activation in DCs and LCs. These effects were mediated primarily through IL-6. The responsible signaling pathway downstream of the IL-6 receptor remains to be identified, since neither inhibition of pSTAT3 nor Raf1 could overcome the glioblastoma-induced phenotypic DC or LC inhibition. In recent years, glioblastoma has become a potential target for DC-based immunotherapeutic approaches. The majority of patients, however, did not show an immunological response, calling for improvement of vaccine strategies. Chapter 8 provides evidence that targeting tumor-derived IL-6 might add to the efficacy of immunotherapy. Of note, the glioma-induced disturbed DC differentiation also affected cytokine release by the stinted DC. Glioma-conditioned and poorly differentiated monocyte-derived DC released lower levels of the cytokine IL-12p70, which, beside its strong immune stimulatory effects, is also recognized for its anti-angiogenic effects. Combined with an observed rise in the production of the pro-angiogenic factor IL-8, our data suggest that blocking IL-6 might prevent the accumulation of immature DC in the glioma microenvironment that would otherwise not only inhibit anti-tumor immunity but also effectively stimulate angiogenesis. Another example of co-regulation of angiogenesis and immune suppression.

Both chapter 7 and 8 investigated the role of pSTAT3, either expressed in tumor cells or in DCs, in tumor immune escape and angiogenesis. In NSCLC we could not find an association between pSTAT3 expression and angiogenesis and the activation status or numbers of tumor-infiltrating DC. In addition, inhibition of pSTAT3 in DC could not overcome the suppressive effects of glioblastoma-derived IL-6. The proposed role of STAT3 as a key regulator in angiogenesis and DC suppression (mostly based on numerous murine studies (29)) could thus not be confirmed in our human studies. Of note, we did

find evidence for an important role of tumor-derived IL-6 in the suppression of DC differentiation in two separate studies: in advanced RCC patients treated with sunitinib, we observed a significant inverse correlation between serum IL-6 levels and MDC-1 frequencies (chapter 6), while glioblastoma-derived IL-6 was identified as the major culprit in the inhibition of LC and DC from $CD34^+$ precursors *in vitro* (chapter 8). These findings are in line with several previous studies on various tumor types, implicating IL-6 as a key suppressive factor in this regard (30). We therefore postulate that systemic IL-6 blockade might be a beneficial addition to any (DC-based) immunotherapeutic approach. Perhaps surprisingly, beside neutralizing anti-IL-6 mAbs, sunitinib might also be a useful tool in this regard, as we observed that the significant inverse correlation between serum IL-6 levels and MDC-1 rates was abrogated upon sunitinib treatment (week 4), but reasserted itself after a subsequent 2-week off-period (week 6, figure 3). This observation is suggestive of a reduced IL-6 responsiveness of the MDC-1 subset due to down-stream TKI activity of sunitinib, although the possibly involved TK targets remain to be identified.

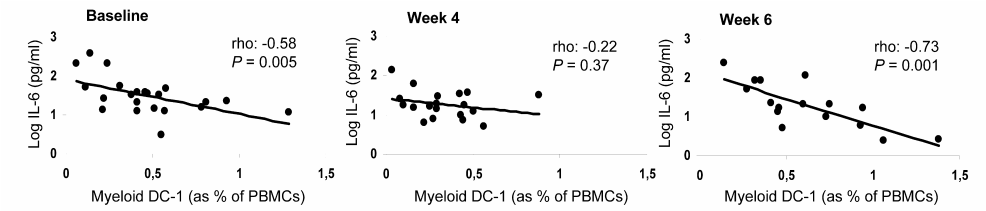

Figure 3 - *Correlation between IL-6 plasma concentrations and myeloid DC-1 frequencies as percentage PBMCs over follow-up in renal cell carcinoma patients treated with sunitinib in a 4-week on/2-week off schedule.*

Besides identifying the specific factors involved in tumor-induced DC suppression and angiogenesis in the particular tumor type to be treated, other considerations should be taken into account when developing a regimen combining anti-angiogenic treatment and immunotherapy. Firstly, anti-angiogenic agents should be evaluated for their potency to overcome tumor-induced defective DC differentiation *in vitro*. Ideally, these anti-angiogenic agents should also be combined with immunotherapeutic approaches and tested in preclinical *in vivo* models for proof of principle. Subsequently, the most effective combined strategy should be tested in a clinical setting. Special attention should be given to the schedule and frequency of the regimen. Added immunotherapeutic approaches may have the greatest potential to increase therapeutic efficacy when given to patients upon simultaneous lifting of tumor-induced immune suppression and normalization of tumor blood flow through the use of angiogenesis/immune modulators, like VEGFR TKI.

Normalized tumor blood flow and abrogated tumor endothelial cell anergy may facilitate tumor infiltration by recruited immune effector cells and thus further contribute to an effective anti-tumor response (2). Secondly, response evaluation in clinical studies combining anti-angiogenic treatment with immunotherapy should focus on survival. As discussed in chapter 2, response evaluation in studies using anti-angiogenic agents is under debate. Uni- or bi-dimensional tumor measurements have been the standard for response evaluation in studies using conventional agents; however, for novel targeted therapies functional imaging may be an alternative. Beside radiologic or morphologic responses, immunomonitoring, especially of in vivo primed cytotoxic T-cells, is widely applied to evaluate response in immunotherapeutic strategies. All these evaluations can serve as surrogate markers for response, but survival should nevertheless remain the end-point of clinical studies.

In conclusion, in this thesis the co-regulation of tumor-induced angiogenesis and immune escape has been explored. Although more (translational) research is needed to fully understand this complicated relationship and to identify additional co-regulators, this thesis provides evidence for the rationale that anti-angiogenic treatment can be a useful addition to immunotherapy. Cediranib has the potential to reduce MSCs and sunitinib increases frequencies of the immune-stimulatory MDC-1 subset. Both might thus provide a window in which increased myeloid immune competence could add to the efficacy of cancer immunotherapy. In the initial clinical evaluation of the efficacy of such combination regimens, one should aim to select a tumor type which has been shown to be responsive to immunotherapeutic approaches, such as glioblastoma, RCC, melanoma or prostate (31-33). An anti-angiogenic agent with proven efficacy for the tumor type of choice, such as sunitinib in RCC (34) or cediranib in glioblastoma (10), could then be combined with a vaccination strategy. Similarly, the vaccination strategy should be selected based on previously reported efficacy data, for instance autologous tumor lysate-pulsed DCs in glioblastoma patients (33) or GVAX in prostate cancer patients (31). Eventually, prospective clinical trials should establish whether immunotherapy can indeed synergize with combined anti-angiogenic treatment.

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